Photoperiodic Regulation on Dio2/3 expression in Middle Aged Siberian Hamsters

Undergraduate Thesis Research

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By

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Abstract

Photoperiodism is the biological ability to measure day length, which provides a reliable cue for time of year. Changes in photoperiod induce seasonal reproductive, metabolic and immunological adaptations to allow animals to anticipate the upcoming season. Siberian hamsters decrease body mass, halt reproductive behaviors, and increase anxiety- and depressive-like responses in short days. Melatonin plays an important role in conveying both circadian and photoperiodic information. Melatonin signaling also regulates hypothalamic levels of thyroid hormones, which has been implicated in the rapid photoperiod mediated changes in physiology. As rodents age, they become unresponsive to short days and continue to maintain reproductive function. The goal of this study is to determine whether changes in gene expression of *Dio2/Dio3* correlate with photoperiodic changes in body mass and behavior of late adult hamsters. Although studies have been conducted in juvenile animals, *Dio* gene expression in their aged counterparts remains unspecified. We predict that middle-aged hamsters (10-12 months) housed in short days will express decreased responsiveness to photoperiod relative to their long day housed counterparts. Middle-aged male Siberian hamsters were individually housed in either short (SD, 8h: 16h) or long (LD, 16h: 8h) day conditions for 4-8 weeks, then tested for anxiety and depressive-like behaviors using the open field, elevated plus, and forced swim tests. Daily food intake and body mass were assessed once per week during the study. At the end of the experiment, hippocampal and hypothalamic tissues were collected for qPCR analysis of *Dio3/Dio2* expression. There was no change in *Dio3* expression between long day and short day housed hamsters, but *Dio2* expression was blunted. This study may provide a novel mechanism by which photoperiodic signals are transduced to regulate body mass and behavioral changes.

Introduction

Organisms respond to several environmental cues to organize annual physiology and behavior, whether it is day length, temperature, or food availability. Changes in day length (or photoperiod) drive major adaptations in physiology and behavior. Small mammals exhibit seasonal changes in reproductive ability and body mass by altering energetic demands.[1] Circadian information is transmitted to the rest of the body through neural and humoral cues. The major humoral signal of time of day and time of year is melatonin. Light stimulates intrinsically photosensitive retinal ganglion cells (iPRGCs), which carry signals along the retinohypothalamic tract to the suprachiasmatic nucleus (SCN). Projections leave the SCN through the paraventricular nucleus and out of the brain to the superior cervical ganglion. They relay back into the brain to the pineal gland which secretes melatonin in the absence of light input.[2] The physiological signal of photoperiod is the nightly melatonin secretion.[3]

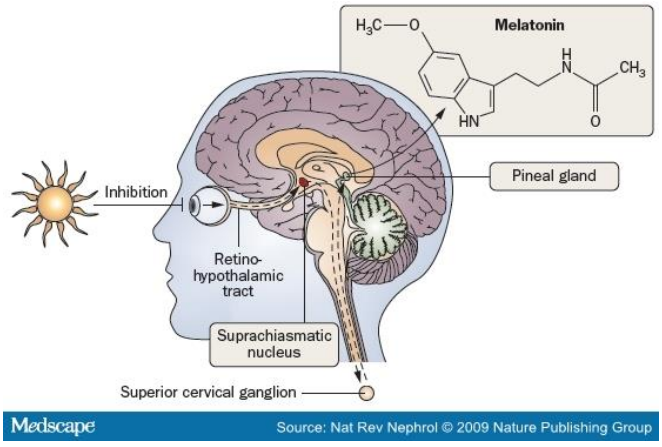

Fig.1. Light Pathway from Eye to Brain [4]

Siberian hamsters (*Phodopus sungorus*) are a useful model species because of their well-characterized responses to photoperiod. The critical photoperiod to maintain the long-day phenotype is > 16 h of light/day.[5] Short day (SD) is characterized by long nights, and since melatonin secretion peaks at nights, there is a longer duration of melatonin secretion signaling

the animals of a changing season (Bernard et al.). SD responses induce “winter-like” day adaptations including decreased body mass, reduced testicular mass and low gonadotrophin and testosterone levels. This decrease in body mass and reproductive behavior allows them to expend less energy and lower metabolic needs.[6] In this catabolic state, short day hamsters may lose up to 40% of their body weight, molting into a white pelage, increase torpor and anxiety-like and depressive behaviors, whereas hamsters housed in long days (LD) are reproductively active, hyperphagic, and display less depressive behaviors.[7]

Physiological adaptations to photoperiod involve complex processes within the neuroendocrine system. Although the hormone melatonin and its role in photoperiodism have been thoroughly studied among mammals and birds, its role in mediating other hormones has not.[8] Various studies have reported on the downstream signaling of melatonin, especially within the hypothalamus. Gene expression in the hypothalamus is regulated in a photoperiodic dependent manner in various species, including sheep, hamsters and quail.[7] Of the genes regulated by photoperiod, thyroid hormone is a key player in the metabolic and reproductive axis.[8]

Deiodinases catalyze metabolic reactions that result in activation or inactivation of thyroid hormones Genes encoding type II and type III deiodinases are selectively expressed in the mediobasal hypothalamus (MBH).[7] Type III deiodinase (Dio3) is responsible for the conversion of thyroxine (T_4) and triiodothyronine (T_3) to their inactive metabolites, thereby protecting tissues from a surplus of hormones.[9] Dio2 or type II deiodinase is an enzyme that catalyzes deiodination of T_4, a prohormone and leads to production of T_3, the active hormone.[10]

Multiple studies have suggested the photoperiodic regulation of thyroid hormone synthesis as an important player in the neuroendocrine axis, [8] one mechanism being through

melatonin induction. Melatonin is known to regulate gonadotrophin secretion. A known location of this activity occurs in the mediobasal hypothalamus (MBH); [11] lesions of the MBH block the inhibitory effects of melatonin on gonadotrophin release.[12] T_3 levels in the MBH are regulated by DIO2 and DIO3 in a photoperiod dependent manner.[13] Studies in birds suggest that expression of the DIO2 gene is light induced, with significant differences in enzyme abundance within MBH between short day and long day. DIO2 is implicated in regulating seasonal reproduction; inhibiting *Dio2* reduces testes growth in Japanese quails housed in long days.[10]

Similar to birds, a link between DIO2 and DIO3 expression in mammals conveys photoperiodic information through melatonin signaling pathways [14]; with upregulation of DIO3 in short days and upregulation of DIO2 in long days, thyroid hormones cast light into different mechanisms of biological rhythms.[15] Unlike most species in which high levels of T_4 are related to a decrease in fat and body mass during short days, Siberian hamsters display a slight decrease in serum levels of T_4 in short days.[16] In one in vivo study on Siberian hamsters, although the expression of DIO2 was unaffected by photoperiod, DIO3 expression increased in hamsters transferred from LD to SD. With a decrease in T_3 levels, a transition in high body mass and active reproduction to low body mass inactive reproduction was possible.[8]

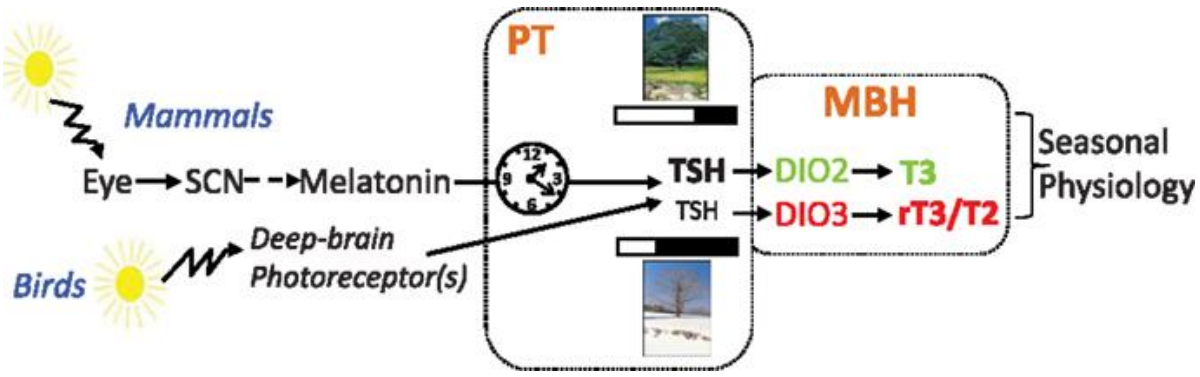

Fig.2. Photoperiodic pathway for mammals and birds [17]

There are aging effects in response to photoperiod in Siberian hamsters. It has been shown in a few studies that aged hamsters between 9 and 12 months are not responsive to photoperiod. Testicular regression is reduced in Siberian hamsters that are more than 12 months old, with no loss in melatonin receptors in the SCN or PT.[23] Although melatonin concentration at night is lower in older hamsters than their juvenile counterparts, the aged hamsters continue to respond to changes in photoperiod that may be due to increased sensitivity to melatonin.[27] However, there are also studies indicating decreased responsiveness to SD conditions. Hamsters that are a year old become unresponsive to SD. A second exposure to SD conditions does not result in pronounced testicular regression or loss in body mass.[28] Therefore, these studies show that aged hamsters are not as responsive to photoperiod as their juvenile counterparts.

This study hypothesizes that the hamsters housed in short day exhibit decreased responsivess to photoperiod because of decreased responsiveness in the *Dio* system. We predict that the aged hamsters will continue to express increased levels of hypothalamic DIO3 gene expression and decreased levels of DIO2 gene expression; however their expression will be dampened. Upregulation of DIO3 implies that decreasing levels of T_3 leading to catabolism resulting in the loss in body mass and reproductive behaviors.

Materials and Methods

Animals

Sixteen (16) middle-aged male Siberian hamsters (*Phodopus sungorus*) were obtained from the in-house breeding colony. The hamsters were individually housed in propylene cages (27 x 7.5 x 13 cm) at an ambient temperature of 21 $\pm$ 1°C and 32% relative humidity. Standard

chow (Harlan Teklad7910; Madison, WI), filtered tap water and cotton nesting were available *ad libitum*. The animals were previously maintained in standard conditions (LD 16h:8h).

Experimental Design

The hamsters were randomly assigned to two lighting conditions of short day (LD 8h:16h) or long day (LD 16h:8h). They were placed in lightproof and ventilated cabinets. The hamsters were maintained in lighting conditions for 8 weeks. Weekly body weights and food weights were measured. After 8 weeks, behavioral testing was conducted and then a week later, brains, spleens, adrenals, and livers were collected. Additionally, fat pads and reproductive tissues were collected and weighed. Pelage scores (integers from 1 to 4; 1 = darkest pelage, 4 = lightest pelage) were also observed before tissue collection to assess responsiveness to photoperiod.

Behavioral Testing

Open field: Hamsters were tested starting at the onset of the dark cycle for activity and anxiety-like behavior using the open field test. The open field test characterizes exploration of a novel environment and indexes overall locomotor activity. Central tendency is the primary measure for anxiety-like behaviors, defined as the proportion of time spent in the center of the open field. Hamsters were allowed to acclimate to the room for a minimum of 20 min before testing. Hamsters were placed in a 40 cm × 40 cm clear acrylic chamber with the center defined as the central 28 cm × 28 cm. Locomotor activity was recorded for 20 min during the open field. Tapes were assessed using Observer software (Noldus, Leesburg, VA) by a condition-blind observer for time in the center versus periphery and total moves during the test.

Elevated Plus: Following open field testing, hamsters were placed in the center of the elevated plus maze with two enclosed arms (50 cm × 10 cm × 40 cm) and two open arms (50 cm × 10 cm). The entire maze was elevated 120 cm off the ground. Animals were allowed to freely explore for 5 min and recorded on video. Video was scored on Observer software (Noldus, Leesburg, VA) by a condition-blind observer for latency to enter the open arms and the percentage of time spent in the open arms.

Forced Swim: Following the elevated plus maze test, hamsters were assessed for depressive-like behaviors on the forced swim test. Mice were placed in a 4000 mL glass beaker filled with 2500 mL of room temperature (22˚C ± 2˚C) water for ten minutes, dried, and returned to their home cage. The video was scored on Observer software (Noldus Corp. Leesburg, VA, USA) by a condition-blind observer for total time floating, swimming, and climbing.

RNA Extraction and cDNA Synthesis

Brains samples were maintained in -80 °C conditions for a week before RNA extraction. They were then halved, with the hypothalamus and hippocampus separated from each brain. The samples were homogenized (Ultra-Turrax T8; IKA Works, Wilmington, North Carolina) and RNA was extracted with TRIzol reagent (Life technologies, Thermo Fisher Scientific Inc.) according to a protocol. The extracted RNA pellet was resuspended in x µL of ultrapure water. A spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific Inc.) was used to determine RNA quality and quantity, followed by cDNA synthesis and qPCR. RNA was reverse transcribed into cDNA using M-MLV Reverse Transcriptase enzyme (Invitrogen, Carlsbad, California).

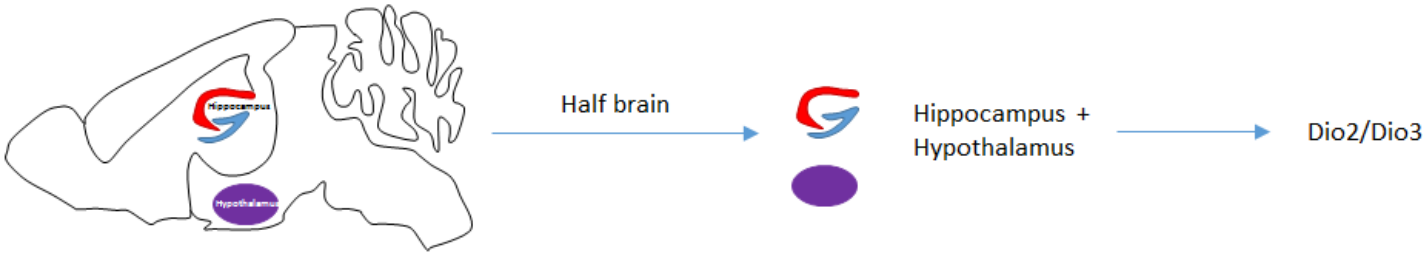

Fig.3. Sectioned Brain for RNA extraction and qPCR (courtesy of Yasmine Cisse)

Quantitative Polymerase Chain Reaction (qPCR)

Dio2 and *Dio3* were individually compared to 18S rRNA expression for relative quantification. qPCR was performed using a 7500 Fast Real-Time PCR System (Life Technologies, Thermo Fisher Scientific Inc.) with SYBR Green chemistry. Designed forwards and reverse primer sequences for *Dio2* and *Dio3* in *Phodopus* sungorus were used in the PCR reaction. The forward primer sequence for *Dio2* was 5'-ACCACCACCTTCCTTTGCAA-3' and reverse sequence was 5'-GCGGAAGGCTGGCAGTT-3'. The forward primer sequence for *Dio3* was 5'- CATSCTGCGCTCYCTGCTGCTTCA-3' and the reverse sequence was 5'-GCGGAAGGCTGGCAGTT-3'. The PCR cycling conditions used for *Dio2* were 95 °C for 10 sec, 58 °C for 30 sec, and 72 °C for 30 sec for 45 cycles. Conditions used for *Dio3* were 95 °C for 10 sec, 62 °C for 30 sec, and 72 °C for 30 sec for 45 cycles. Expression for *Dio2* and *Dio3* was normalized to 18S rRNA expression and calculated by comparison to a delta delta CT.

Statistical Analyses

Change in body mass with time as a within subject variable was assessed using a repeated measures ANOVA. Comparisons of weight gain between groups were conducted using a one - way ANOVA with lighting condition as between subject factor. Post hoc tests of statistically

significant groups were performed using Tukey's HSD test. Differences between means were considered statistically significant when set at $p \leq 0.05$ for all analyses. Animal 3 was considered an outlier for the food intake and body mass measurements.

Results

Body Mass, Pelage Scores, and Food Intake in SD and LD Conditions

Body mass did not significantly differ between the hamsters housed in SD and LD conditions after 8 weeks of photoperiodic exposure ($p > 0.05$). There was also no statistically significant difference in pelage scores (integers from 1 to 4; 1 = darkest pelage, 4 = lightest pelage) between hamsters in the two lighting conditions ($p > 0.05$). Differences in total food intake between hamsters housed in SD and LD conditions were not statistically significant ($p > 0.05$).

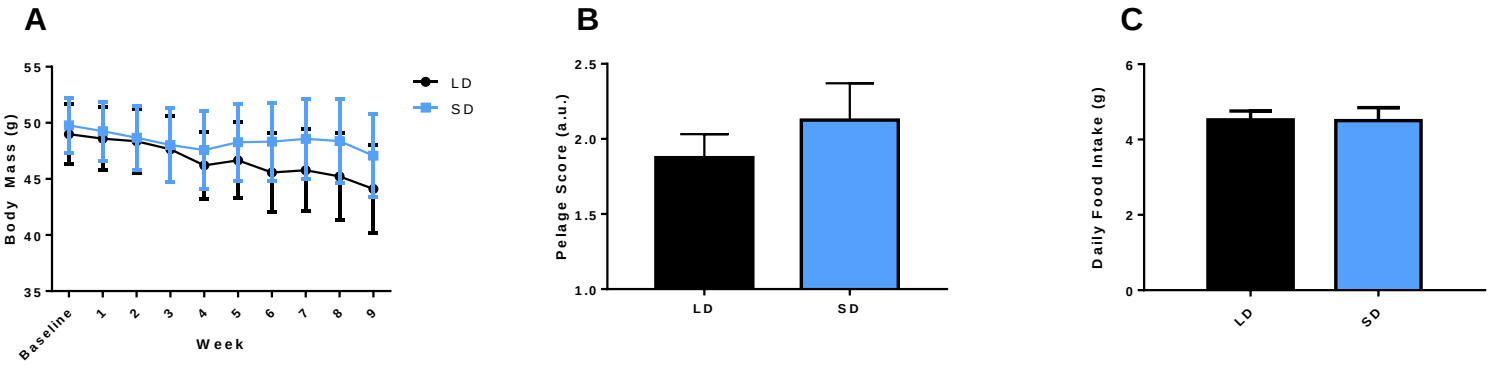

Fig. 4. Mean ± SEM (A) Body mass over time, (B) Pelage score, and (C) Food Intake

Effect of SD and LD Conditions on Reproductive Organ Mass

Testes mass as a percentage of body mass was decreased in SD housed hamsters ($F_{1,14}$ = 8.419, $p < 0.05$). Values in % body mass of seminal vesicles and gonadal fat pads were comparable among hamsters in both the lighting conditions ($p > 0.05$ in all comparisons).

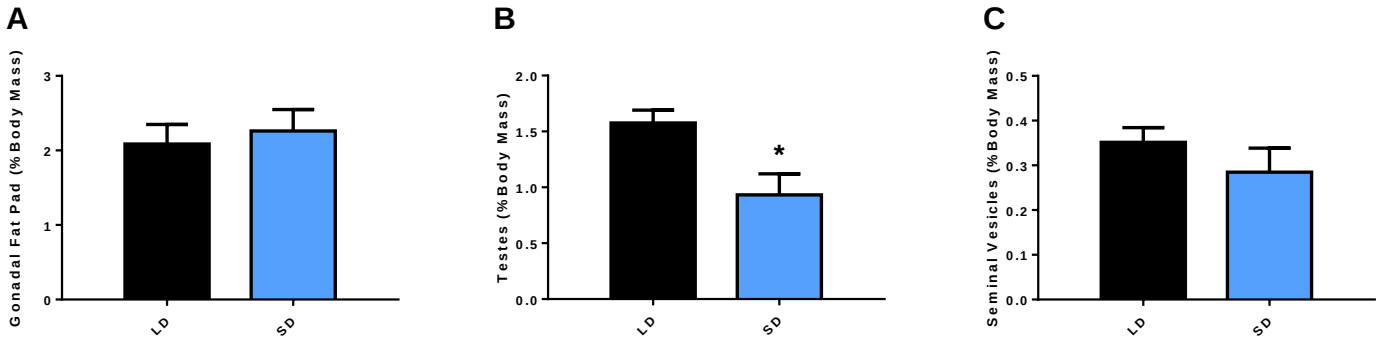

Fig. 5. Mass as % Body Mass in (A) Gonadal Fat Pad, (B) Testes, and (C) Seminal Vesicles

Behavioral Responses to SD and LD conditions

The forced swim test yielded statistically significant results. The mean time spent floating by SD housed hamsters was greater than their LD counterparts ($F_{1,14}$ = 3.254, $p < 0.01$). SD housed hamsters spent less time swimming, with the latency to float occurring earlier. There was no difference in the open field test between SD and LD housed hamsters ($p > 0.05$). Times spent in the center and open arms of the elevated plus test were comparable in the hamsters in the two lighting conditions ($p > 0.05$).

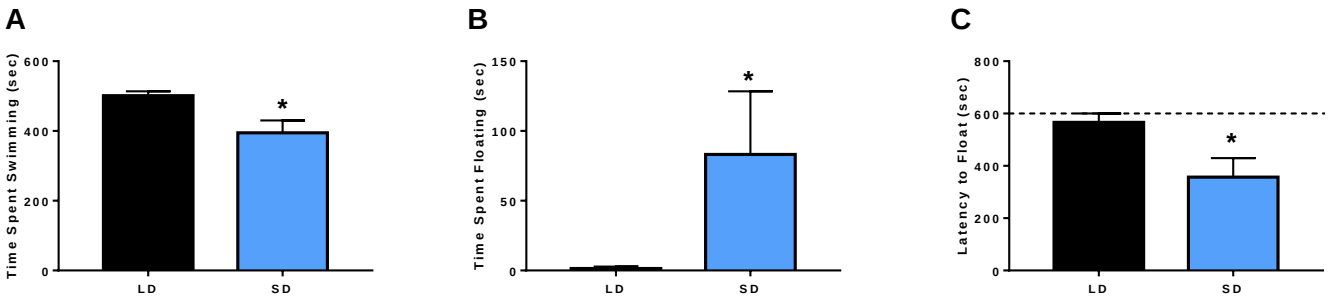

Fig.6. Mean ± SEM (A) Time spent swimming (in sec), (B) Time spent floating (in sec), and (C) Latency to float (in sec)

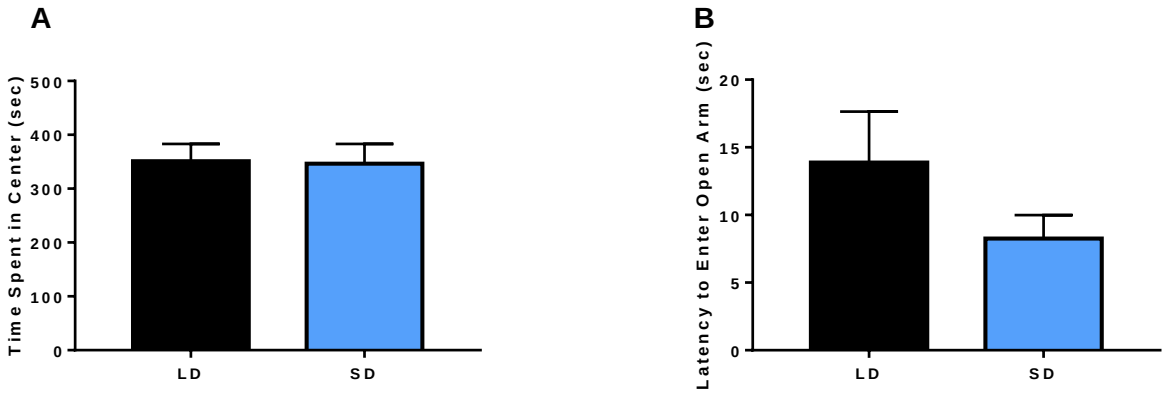

Fig 6 Mean ± SEM (A) Time spent in center (in sec), and (B) Latency to enter open arm (in sec)

qPCR results for Dio2 and Dio3 expression

Dio2 expression in the hypothalamus decreased in SD housed hamsters ($F_{1,14} = 8.340$, $p < 0.05$). There was no significant change in expression of hypothalamic *Dio3* between SD and LD housed hamsters. Photoperiod seemed to have little to no effect on hippocampal levels of *Dio3* and *Dio2* among the hamsters.

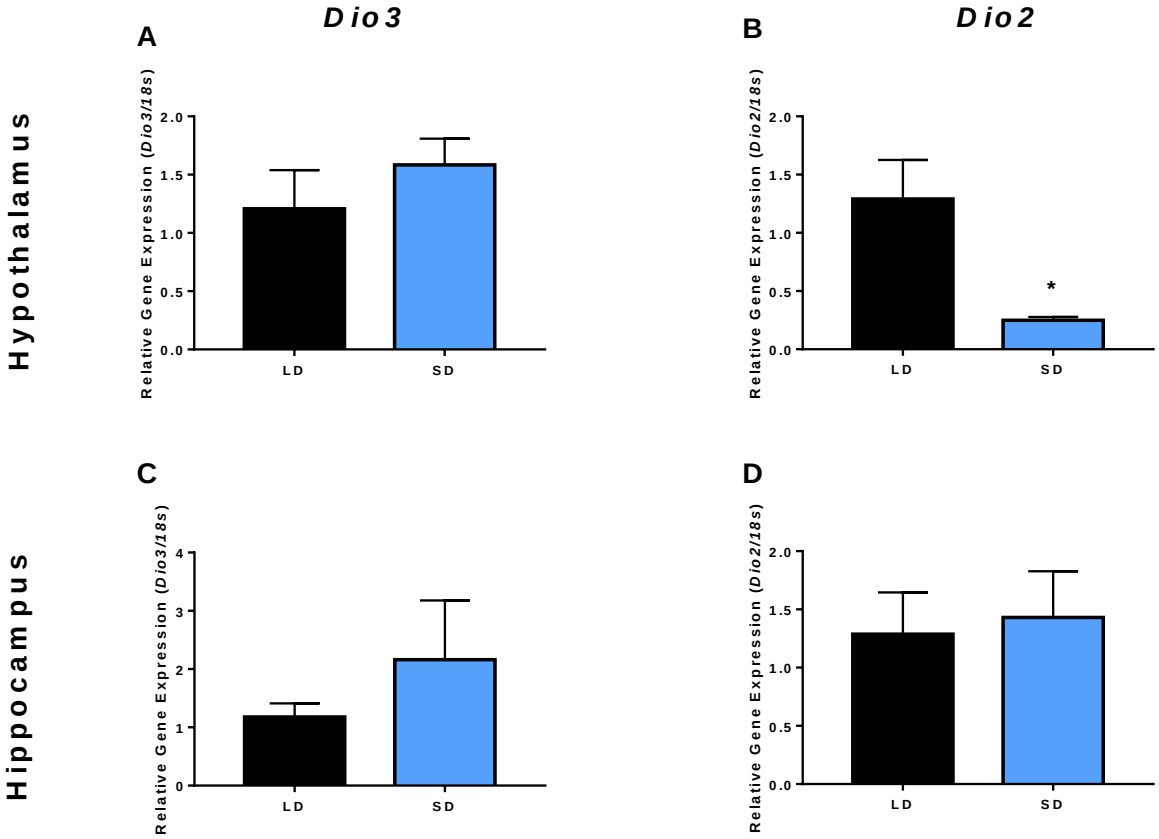

Fig. 7. Expression of *Dio3* (A and C) and *Dio2* (B and D) in Hypothalamus and Hippocampus

Discussion

There was no significant difference in body mass, pelage score, and food intake between the hamsters housed in SD and LD conditions. These results show the dampened effect of photoperiod on aged mice because SD housed juvenile hamsters typically lose body weight as a result of decreasing their energy requirements.[18] Instead of the white pelage that SD housed hamsters exhibit, the middle aged hamsters in this study failed to display a change in fur color.[19] There was no effect of photoperiod on pelage as the mean SD pelage score was 2.1250 ± 0.69,

with a score of 4 equivalent to a “winter-like” phenotype common in SD housed hamsters. Exposure to short days decreases food intake in hamsters as a result of fewer abundance of intestinal bacteria, whereas hamsters with more intestinal proteobacteria increased body and fat masses.[20] There was however no difference in food intake among hamsters housed in SD or LD conditions

Testes mass relative to body weight decreased in SD housed hamsters. Gonadal atrophy has been studied by injecting varying doses of melatonin into the brain, with injections into the SCN resulting in the most distinct inhibition of testis growth.[21] This pattern shows that the increasing levels of melatonin secreted in short day photoperiod leads to a decrease in reproductive activity. SD housed hamsters are also expected to have less gonadal fat pad mass, with a depletion in white adipose tissue pads.[22] This was not seen in the hamsters receiving SD lighting conditions, as fat pad mass as a percent of body mass was comparable between the SD and LD housed hamsters. Taken together, with the exception of adjusted testes mass, the SD hamsters in the study did not attain the typical SD phenotype.

It has been hypothesized that response to photoperiod diminishes with age in hamsters.[23] Testes and body mass were less pronounced in SD housed hamsters aged between 9 and 12 months. This parallels some of the results found in this study as hamsters were aged 10 months at the beginning of the experiment and 12 months by the end. This phenomenon in aged hamsters gives rise to questions at the proximate levels of analysis about the photoperiod induced melatonin pathway and the molecular mechanisms behind changes to body mass and behavior. Thyroid hormones and their enzymes come into play in attempting to explore the pathways. Since T_3 levels are regulated by deiodinases, which in turn vary according to melatonin levels, deiodinase gene expression was looked into in this study.[13] Hypothalamic Dio3 expression is

increased in hamsters housed in SD, almost a 25 fold increase relative to LD counterparts.[14] Compared to these findings, the results in this study found that hypothalamic *Dio2* expression in SD housed hamsters decreased while there were no differences in hypothalamic *Dio3* expression between the two groups. This can be a possible explanation of why there was no change in body mass between SD and LD housed hamsters. The results provide further evidence for the regulation of thyroid gene expression by both photoperiod and melatonin. It has also been suggested that *Dio3* is more closely linked to body mass and photoperiod responses as a result of rapid induction of changes through epigenetics. Gonadal recrudescence occurs after 20-30 weeks of exposure to SD conditions.[24] The mechanisms behind this spontaneous photorefractoriness are attributed to epigenetics, especially methylation. As a result of decreased methylation in the promoter region of *Dio3*, there is increased gene expression leading to SD responses in hamsters. Remethylation of the promoter decreased Dio3 expression occurs during photorefractoriness, thereby initiating reproductive activity.[1] There may be changes in methylation of the *Dio3* promoter that is preventing expected behavior in aged SD hamsters.

The hippocampus is studied in terms of changing behavior in response to SD photoperiod. SD housed mice have decreased brain mass and hippocampal volume, indicating deficits in spatial learning and spine density.[25] Although the forced swim test produced statistically significant results, the open field and elevated plus did not. Just as aged hamsters are less responsive to photoperiod in terms of body mass, behavior may also not be effectively mediated by photoperiod. The comparable results in *Dio3* and *Dio2* expression in the hippocampus between SD and LD housed hamsters might help explain this effect on behavior.

On an ultimate (adaptive) level of analysis, the results of the study are not unexpected. Siberian hamsters only survive about a year in the wild.[26] If hamsters are born during the brief

Siberian summer, then reproductive function is suppressed by short days and reestablished the following spring. However, the odds of surviving a second winter are small so male hamsters may have been selected to ignore photoperiodic information and maintain reproductive function in case of conditions which are sufficiently mild for successful winter mating.[26] The *Dio3* and *Dio2* expression data of this study may provide the physiological basis for this adaptive change in photoperiodic responsiveness.

Future Directions

This study aimed to investigate the molecular mechanisms behind the lack in response of aged hamsters to photoperiod. The dampened expression of hypothalamic Dio3 expression might help explain the weak effect of photoperiod on body mass in SD housed hamsters. These data provide a stepping stone to further explore the role of methylation in thyroid genes and the role of microRNAs in the regulation of responses to photoperiod. The concepts can be applied to the study of seasonal affective disorder in humans and can lead to a better understanding of the intricate mechanisms behind biological rhythms.

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